

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



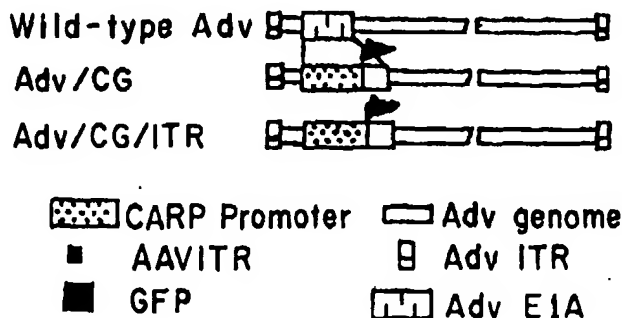
INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification 7 : C12N 15/86, A61K 48/00</p>	<p>A1</p>	<p>(11) International Publication Number: WO 00/15821 (43) International Publication Date: 23 March 2000 (23.03.00)</p>
<p>(21) International Application Number: PCT/US99/20730 (22) International Filing Date: 10 September 1999 (10.09.99) (30) Priority Data: 60/099,960 11 September 1998 (11.09.98) US (71) Applicant: THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 9500 Gilman Drive Mail Code 0910, La Jolla, CA 92093-0910 (US). (72) Inventors: CHIEN, Kenneth, R.; 6232 Calle Vera Cruz, La Jolla, CA 92037 (US). WANG, Yibin; 4142 Caminito Lita, San Diego, CA 92122 (US). EVANS, Sylvia; 2281 Via Apilia, Del Mar, CA 92014 (US). (74) Agents: MUSICK, Eleanor, M. et al.; Brown, Martin, Haller & McClain, 1660 Union Street, San Diego, CA 92101-2926 (US).</p>		<p>(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</p> <p>Published With international search report.</p>

(54) Title: RECOMBINANT ADENOVIRUS FOR TISSUE SPECIFIC EXPRESSION IN HEART

(57) Abstract

The present invention relates to a human type-5 recombinant adenovirus vector for achieving cardiac restricted transcription involving utilization of the cardiomyocyte-restricted cardiac ankyrin repeat protein (CARP) promoter with inclusion of the inverted terminal repeat sequences from human adeno-associated virus (AAV). Using green fluorescent protein (GFP) as a marker gene, the recombinant adenovirus vector (Ad/CG/ITR) is shown to direct transgene expression to myocardial tissue *in vivo* and *in vitro* in mouse models.



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LJ	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

RECOMBINANT ADENOVIRUS FOR TISSUE SPECIFIC EXPRESSION IN HEART

This application claims the benefit of priority of United States Provisional Application Serial No. 60/099,960, filed September 11, 1998,
5 which is incorporated herein by reference in its entirety.

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

This invention relates generally to a recombinant adenoviral vector construct and to methods for the study of gene function and gene therapy
10 for heart disease and more specifically to methods of targeting tissue specific expression of a given transgene in cardiac tissue through use of inverted terminal repeat sequences from human adeno-associated virus.

BACKGROUND INFORMATION

Cardiovascular gene therapy represents a novel approach to the
15 treatment of inherited and acquired heart disease. Gene transfer to the heart would allow for the replacement of defective or missing cellular proteins that are responsible for proper cardiac function. The control of *in vivo* cardiac function represents a complicated interplay between multiple genes, varied cell types, and environmental stimuli but the elucidation of
20 this interplay remains dependent on a more complete understanding of the changes that occur at the molecular and cellular levels. Traditionally, the majority of human gene therapy protocols have relied on the *ex vivo* application of the therapeutic gene, through the introduction of a retroviral vector, to the affected cells or tissue. Because the *ex vivo* method of
25 gene therapy depends on the removal from and reintroduction to the body of the target cells, the treatment of inaccessible or sensitive organs or tissue poses a major dilemma. The alternate strategy of direct *in vivo*

delivery of therapeutic genes to the target cells represents a preferable method of gene therapy.

Targeted gene expression in somatic tissues is essential for both gene therapy and *in vivo* analysis of gene function, mainly through the substitution of an affected gene, using a safe and effective delivery system for the therapeutic gene. To date, recombinant adenoviruses have replaced the retrovirus as an efficient gene delivery vector for a variety of cell types and tissues (Yeh, *et al.*, FASEB J 11, 615-23, 1997). Adenovirus vectors are highly efficient in the genetic modification of nondividing human cells and have the capacity to carry long segments of genetic information. The hurdle in using adenovirus as gene "delivery systems" is that when an adenovirus is administered to a patient to aid in the delivery of genes to specific cells, the patient's immune system may react against the virus. To overcome this hurdle, modifications have been made to make the adenoviral vector safer, less toxic to the cells and less likely to stimulate an immune response. This has involved removing the E1 region of the adenovirus gene which prevents the ability of the virus to express its own proteins required for making viral particles. In place of the E1 region, a therapeutic transgene can be inserted. The efficiency of this kind of exogenous gene delivery and subsequent expression can be high, as it does not normally integrate into the host genome, and it has a minimal effect on intrinsic host cell function (Baldwin, *et al.*, Gene Ther. 4, 1142-49, 1997). However, while adenoviral vectors are capable of producing high levels of transgene expression, their capacity to infect and program transgene expression in large numbers of cells and tissue, including the liver and lungs, poses limitations. As a result of this high level of transient infectivity, methods have been undertaken to direct transgene expression to specific tissues or areas of the body. For cardiac tissue, a number of attempts have been reported utilizing recombinant adenoviruses to achieve transgene expression in the heart through either intra-myocardial or intra-coronary injection (Brody, *et al.*, Ann. N.Y. Acad.

Sci. 716, 90-101, 1994; Barr, *et al.*, Gen Ther. 1, 51-8, 1994; Kypson, *et al.*, J. Thorac. Cardiovasc. Surg. 115, 623-30, 1998). While direct injection of viral particles into the myocardium or cardiac cavity have been shown to be more efficient for gene delivery to the myocardium, infection and transgene expression also occurs in non-cardiomyocytes, which causes speculation that any specificity of transgene expression that exists is achieved by targeted delivery rather than restricted transcription (Kass, *et al.*, Gene Ther. 1, 395-402, 1994; Kass, *et al.*, Methods Cell Bio. 52, 423-37, 1997). As a result, ectopic expression, particularly in liver and other tissue, remains a significant limitation for the generalized use of recombinant adenoviruses for gene transfer to specific cell types within the cardiovascular and other organ systems.

In most recombinant adenoviral vectors, the E1a region of the adenovirus genome, which encodes the protein with properties for transcriptional regulation, is deleted and replaced by a minigene "cassette" that typically includes a promoter of choice, the transgene coding region, and a polyadenylation signal (Yeh, *et al.*, FASEB J 11, 615-23, 1997). One possible approach to achieve tissue-specific transgene expression using adenoviruses is to employ cellular gene promoters that possess cell-type specificity at the transcriptional level, rather than commonly used viral gene promoters that have a high level of expression, but lack tissue specificity. In the past, a number of studies have utilized different cell promoters to achieve targeted transgene expression in various tissues, including smooth muscle (Kim, *et al.*, J. Clin. Invest. 100, 1006-14, 1997), pancreas (Duseti, *et al.*, J. Biol. Chem. 272, 5800-4, 1997), endothelium (Morishita, *et al.*, J. Biol. Chem. 270, 27948-53, 1995), lung (Strayer, *et al.*, Am. J. Respir. Cell Mol. Bio. 18, 1-11, 1998), and several kinds of tumors (Su, *et al.*, Proc. Natl. Acad. Sci. USA 94, 13891-6, 1997; Siders, *et al.*, Cancer Res. 56, 5638-46, 1996). Similar attempts using cardiac-specific promoters such as the myosin light chain-2 (MLC-2v) and the alpha-myosin heavy chain (α -MHA) promoters, in the context

f ad noviruses, however, have not been wholly successful in providing tissue-restricted gene expression *in vivo* (Kim, *et al.*, J. Clin. Invest. 100, 1006-14, 1997). These results suggest that adenoviral genomic sequences surrounding the deleted E1a region may be responsible for at least partial specificity of the adjacent cellular promoter. It has also been suggested that sequences around the E1a region may contain negative regulatory elements that act in modulating the specificity and activity of a cellular promoter (Shi, *et al.*, Hum. Ther. 8, 403-10, 1997). This undesirable property of adenoviral vectors has limited their application, especially in the context of *in vivo* studies where tissue specific expression of the transgene is required.

Thus, the need remains for a transgene expression system utilizing recombinant adenoviral vectors that are tissue specific for use in *in vivo* and *in vitro* gene therapy and gene function analysis for both neonatal and adult subjects. The present invention satisfies this need and provides related advantages as well.

SUMMARY OF THE INVENTION

The present invention provides a human type-5 recombinant adenovirus vector to achieve cardiac restricted transcription in both neonatal and adult subjects utilizing the cardiomyocyte-restricted cardiac ankyrin repeat protein (CARP) promoter in cooperation with the inverted terminal repeat (ITR) sequences from human adeno-associated virus (AAV). Such a combination is effective in achieving cardiac tissue-specific transcription of the transgene both *in vitro* and *in vivo*.

The invention further provides a method to achieve tissue targeted expression of a given transgene in cardiac tissues in both neonatal and adult subjects. Such a method has significant applications in both gene function studies and gene therapy for inherited and acquired heart diseases.

BRIEF DESCRIPTION OF THE DRAWINGS

Figur 1 shows the constructs of recombinant adenovirus vectors. All recombinant adenovirus vectors were generated through homologous recombination between pJM17 plasmid DNA and the specific shuttle
5 plasmid DNA in 293 cells.

Figure 2 shows a Northern-blot analysis of the relative cell-type specific transcription of GFP in cultured cells following adenovirus infection. RNA from uninfected, control and infected cardiac myocytes were subject to Northern-blot analysis using GFP coding sequences as a
10 probe and normalized by hybridization signals for GAPDH mRNA.

Figure 3 shows a Southern-blot analysis of the relative cell-type specific transcription of GFP in cultured cells following adenovirus infection. DNA from control or infected cells were digested with NotI and XhoI restriction enzymes and the GFP expression was detected at
15 approximately 3.0 kb size for Adv/CMV/GFP and 760 bases for Adv/CG/ITR.

Figure 4 shows a Northern-blot analysis of the level of GFP transcription in mouse heart and liver following intra-cardiac injection of adenovirus vectors.

20 DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

The present invention provides a means for achieving cardiac restricted transcription of a transgene in both neonatal and mature cardiac tissues through the use of a recombinant adenoviral gene delivery vector which is engineered to contain a cardiomyocyte-restricted CARP promoter
25 in conjunction with inverted terminal repeat sequences from human adeno-associated virus, the sequences of which are incorporated herein by reference. In the construction of adenovirus vectors, it is most common to delete the majority of the E1a and E1b regions of the serotype 5 adenovirus gene to prevent replication of the adenoviral DNA. A
30 prototypical vector is constructed by inserting the desired exogenous

genetic information, including the left hand end inverted terminal repeat (ITR), signal enhancers, promoters for the expression of the desired exogenous gene, and a polyadenylation signal, into the former E1 position of the adenovirus. Fu, *et al.* (*Nat. Biotechnol.* 16, 253-7, 1998)

- 5 incorporated herein by reference, have reported an unusual property of the inverted terminal repeat (ITR) sequences, specifically of adeno-associated virus (AAV). Adeno-associated viruses are satellite viruses derived from replication-deficient parvovirus and most often found in association with adenovirus or herpes simplex virus. The wild-type AAV is non pathogenic
- 10 and can site specifically integrate into a host genome, can transduce nondividing cells, and does not induce an immune response which could destroy the transduced cells. Fu, *et al.* have shown that the inclusion of both the left and right end segments of the AAV-ITR sequences imparts the ability to enhance the level as well as tissue specificity of the
- 15 transgene expression using viral gene promoters or tissue-specific cellular gene promoters in developing *Xenopus* embryos. Further, Philip, *et al.* (*Mol. Cell Bio.* 14, 2411-8, 1994) have demonstrated that the inclusion of both the left and right end AAV-ITR sequences in mammalian plasmid constructs results in the enhancement of efficiency and stability of
- 20 transgene expression. In the context of a recombinant adenovirus vector, inclusion of both the left and right end ITR sequences from adeno-associated virus has the ability to enhance tissue specificity of the exogenous transgene expression when a cardiac restricted promoter is utilized.

- 25 In order to achieve targeted gene expression in the cardiac tissue, the 213 base pair, 5' flanking promoter fragment of the CARP gene was selected to direct the transgene expression. Three separate lines of transgenic mice were created which harbored various CARP promoter/ β -galactosidase reporter genes for the purpose of studying this 5' flanking
- 30 CARP promoter. CARP, a cardiac ankyrin repeat protein, is a putative downstream regulatory gene in the homeobox gene *Nkx2-5* pathway

which regulates the expression of the ventricular myosin light chain-2 (MLC-2v) gene (Zou, *et al.*, *Development* 124, 793-804, 1997). Studies have identified an essential GATA-4 binding site in the proximal upstream regulatory region of the CARP gene and cooperative transcriptional

5 regulation mediated by Nkx2.5 and GATA-4. This cooperative regulation is dependent on the binding of GATA-4 to its cognate DNA sequence in the promoter, which suggests that Nkx2.5 may exert its control on the CARP promoter, at least in part through GATA-4. As used herein, the term "homeobox gene Nkx2-5" refers to the murine homologue of

10 *Drosophila* gene *tinman* which has been previously shown to be required for heart tube looping morphogenesis and ventricular chamber-specific myosin light chain-2 expression during mammalian heart development. Ventricular myosin light chain-2 (MLC-2v), one of the earliest markers of ventricular regionalization during mammalian cardiogenesis, has been the

15 subject of numerous studies seeking to identify the molecular pathways that guide cardiac ventricular specification, maturation and morphogenesis. These studies have identified a 28 base pair HF-1a/MEF-2 *cis*-element in the MLC-2v promoter region which appears to confer the cardiac ventricular chamber-specific gene expression during cardiogenesis

20 as well as showing that the ubiquitous transcription factor YB-1 binds to the HF-1a site in conjunction with a co-factor. Moreover, data further indicates that regulatory elements within the 5' flanking region of the CARP gene are capable of directing region-specific (atrial vs. ventricular and left vs. right) transgene expression in the heart. The 213 base pair

25 sequence element in the 5' flanking region of the CARP gene appears to be sufficient to confer conotruncal-specific transgene expression.

CARP forms a physical complex with YB-1 in cardiac myocytes and endogenous CARP seems to be localized in the cardiac myocyte nucleus. Zou, *et al.* (*Development* 124, 793-804, 1997) have demonstrated that

30 CARP can negatively regulate HF-1-TK minimal promoter activity in an HF-1 sequence-dependant manner in cardiac myocytes as well as displaying

transcriptional inhibitory activity when fused to a GAL4 DNA-binding domain in both cardiac and non-cardiac cells. Analysis using a standard Northern-blot protocol indicates an enriched level of CARP mRNA in the myocytes of cardiac tissue, and to a lesser degree in skeletal muscle, and
5 that endogenous CARP expression can be upregulated in heart and other-tissue upon induction of cytokine activity (Chu, *et al.*, *J. Biol. Chem.* 270, 10236-45, 1995; Jeyaseelan, *et al.*, *J. Biol. Chem.* 272, 22800-8, 1997).

Cytokines play a critical role in the control and maintenance of the
10 signaling pathways that regulate mammalian physiology in multiple organ systems. Their widespread importance is reflected in the extensive tissue distribution of cytokine networks, where a deficiency in cytokine signaling components can result in multiple organ defects. In a study by Hirota, *et al.* (*Cell* 97, 189-198, April 16, 1999) incorporated herein by reference,
15 researchers explored the role of IL-6 related cytokines in the pathogenesis of cardiac failure, which is the leading cause of combined morbidity and mortality in the United States and other developed countries. In response to chronic increases in blood pressure and blood volume overload, as is common in myocardial injury, the heart responds by becoming enlarged in
20 order to maintain normal cardiac function, a process known as compensatory hypertrophy. CT-1, a member of the IL-6 cytokine family, can activate the onset of myocyte hypertrophy in vitro and has been shown to be vital as a potent myocyte survival factor in cardiac muscle cells by blocking the onset of cardiomyocyte apoptosis. There is further
25 evidence that the presence of cytokine receptor gp130 expression in cardiac myocytes can lead to compensatory cardiac hypertrophy, thus delaying the onset of cell apoptosis and ultimately, heart failure. A deficiency in the gp130 cytokine receptor signaling pathway often results in severe cardiac defects in developing embryos possibly leading to an
30 early lethality *in utero*. A therapeutic strategy of introducing the transgene coding region of gp130 directly into the embryonic heart cells

using the tissue specific adenoviral vector delivery system of the present invention, while still *in utero*, may be a viable treatment option. Similarly, introduction of the gp130 gene into mature cardiac myocytes under constant biomechanical stress, through attachment to the cardiac specific

5 CARP promoter of the present invention, may initiate expression of the - gp130 cytokine receptor pathway, resulting in enhanced cardiac compensatory hypertrophy, offsetting cardiomyocyte apoptosis, and thus averting cardiac failure.

Generation of recombinant adenovirus vectors

10 The recombinant adenovirus vector of the present invention was constructed through homologous recombination between shuttle plasmid DNA containing the transgene and pJM17 plasmid DNA containing the entire genome of the human type-5 adenovirus, the method of construction described by Wang, et al., *J. Biol. Chem.* 273, 2161-8,

15 1998, for the generation of Adenovirus/CMV vectors. The *E. coli* host containing plasmid pJM17 that includes DNA of the entire genome replication defective human type-5 adenovirus has been deposited as ATCC Accession No. _____ in the American Type Culture Collection, 10801 University Blvd., Manassas, Virginia 20110-2209, U.S.A., under

20 the terms of the Budapest Treaty on the International Recognition of Deposits of Microorganisms for Purposes of Patent Procedure and the Regulations promulgated under this Treaty. Samples of the deposited material are and will be available to industrial property offices and other persons legally entitled to receive them under the terms of the Treaty and

25 Regulations and otherwise in compliance with the patent laws and regulations of the United States of America and all other nations or international organizations in which this application, or an application claiming priority of this application, is filed or in which any patent granted on any such application is granted.

The shuttle plasmid, pAdv/CARP, was assembled with a 2.5 kilobase CARP promoter, excised from the 5' flanking region of the CARP gene and inserted between the Bam HI and Xho I sites of pXCJL.2. (The *E. coli* host containing plasmid pJM17 including DNA of the entire genome of the human type-5 adenovirus containing the insert murine CARP promoter sequence has been deposited as ATCC Accession No. _____.) The resulting construct was shown to be sufficient to confer cardiac-restricted marker gene expression in cultured cells and transgenic mice. (See Zou, *et al. (Development 124, 793-804, 1997).*)

10 With the elucidation of CARP function, this 2.5 kilobase CARP promoter was thus used to generate an adenovirus/CARP/marker construct, using a green fluorescent protein (GFP) gene as a visual reporter for identification of adenovirus/CARP promoter activity following *in vitro* and *in vivo* administration of the adenovirus construct. To
15 construct the reporter gene, GFP coding sequences were excised from pEGFP-N1 (Clontech, CA) through Bam HI and Afl III digestion, and inserted into the Xho I site of pAdv/CARP to generate pAdv/CG. The resulting recombinant adenovirus was designated Adv/CG.

In order to determine whether inclusion of AAV ITR sequences in
20 the adenovirus genome has the ability to enhance tissue specific expression of the transgene, the DNA fragment containing the CARP promoter and GFP coding sequences was removed from pAdv/CG through Bam HI and Sal I digestion and subsequently inserted into the Xho I site of the pAdv/AAV plasmid, which is derived from pXCJL.2 containing two
25 copies of the AAV ITR sequence. The resulting plasmid, pAdv/CG/ITR, was used to generate a recombinant adenovirus, designated as Adv/CG/ITR, using transformation techniques known to those in the art. Figure 1 provides a diagrammatic representation of the recombinant adenovirus constructs. All recombinant adenovirus vectors were plaque-
30 purified using standard methods and analyzed by PCR for the presence of the transgene in the viral genome. High titer viral stocks were prepared

by a single ultracentrifugation on a CsCl gradient as described by Wang, et al., *J. Biol. Chem.* 273, 2161-8, 1998, the technique well known in the art.

Cardiomyocytes and cardiac fibroblast culture and adenovirus infection

- 5 To establish the cardiac tissue specificity of the adenoviral vector of the present invention, primary ventricular myocytes and cardiac fibroblasts were prepared from 1 - 2 day old Sprague-Dawley rats using a Percoll gradient method as described by Iwaki, et al., *J. Biol. Chem.* 265, 13809-17, 1990. Cardiac fibroblasts were isolated from the upper band of the
- 10 Percoll gradient, and subsequently plated in high glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Myocytes were isolated from the lower band of the Percoll gradient and subsequently plated in 4:1 Dulbecco's modified Eagle's medium; 199 medium, 10% horse serum and 5% fetal bovine serum. The cardiac
- 15 fibroblasts and myocytes were infected with the recombinant adenovirus at varied multiplicity of infection (M.O.I.) 24 hours after isolation and were then incubated for an additional 48 hours before being subject to DNA, RNA, and fluorescent photomicroscopic analysis.

RNA and DNA analysis

- 20 RNA samples were prepared from cultured cells and mouse tissues using RNAzol B solution according to the manufacturer's protocol (TEL-TEST, Texas). Northern blot hybridization was performed according to a standard protocol, familiar to those of skill in the art, using GFP coding sequences to generate a P³² labeled probe. Total DNA, purified from
- 25 cultured cells and mouse tissues, were prepared using the protocol as directed by a Purogene DNA isolation kit, and then digested with the restriction enzymes Xho I/Not I for Southern blot analysis using the same P³² labeled GFP coding sequence probes as used in the Northern blot hybridization.

In vivo adenoviral injection into neonatal mouse heart

Using the procedure of high efficiency, long term expression via adenoviral vector injection into neonatal mouse as described by Brody, et al., *Ann. N.Y. Acad. Sci.* 716, 90-101, 1994, 1-day old mouse neonates
5 were anesthetized by hypothermia at 4 °C for 2 minutes. 10 μ l of viral – solution, containing 2×10^9 viral particles, were injected directly into the cardiac cavity using a flame stretched capillary tube mounted on a micromanipulator. Flashback of pulsatile blood in the capillary tube gave positive indication of correct intracavitary placement. The subject
10 neonatal mice were allowed to recover by rewarming at room temperature and were then placed back with the mother for a 48 hour period. At the end of the 48 hours, the neonatal mice were sacrificed, and the heart and liver were removed from the body for DNA, RNA and fluorescent photomicrographic analysis.

15 Mouse embryo culture and microinjection of adenovirus vector

The preparation of rat serum was by the method as described by Cockroft, et al., *Dissection and Culture of Post-Implantation Embryos*, 1990 (IRL Press, Oxford, England). Whole mouse embryos were cultured according to the method of Sturm and Tam, *Methods Enzymol.* 225, 164-
20 90, 1993. As per the protocol, timed pregnant female mice were sacrificed by cervical dislocation. The uterus was dissected from the body and rinsed in phosphate buffered-saline (PBS) to remove any residual blood and then transferred to a sterile receptacle containing PB1 media (137 mM NaCl; 2.7 mM KCl; 0.5 mM $MgCl_2$; 8.04 mM Na_2HPO_4 , 1.47
25 mM KH_2PO_4 ; 0.9 mM $CaCl_2$; 0.33 Na pyruvate; 1g/L glucose; 0.01g phenol red, pH 7.35; 100 ml/L streptomycin; 100 U/ml penicillin; all reagents from Sigma Biochemicals, St. Louis, MO.). Embryos of 11 days post coitum (E11) were dissected from the uterus and the decidua and Riechert's membrane removed. The embryos were separated from the
30 yolk sac and amnion, which had been left attached during dissection to

ensur continuity of the vessels connecting the embryo to th yolk sac or the umbilical vessels from the embryo to the placenta. The isolated embryos were then transferred to pre-equilibrated media (consisting of 50% rat serum which was continuously gassed (95% O₂, 5% CO₂)) in
5 roller culture bottles placed on a rocker table and incubated at 37°C. -
After one hour in culture, the embryos were placed in a petri dish and microinjected into the left ventricle using a 6 µm diameter glass pipette. The micropipettes had been previously prepared using a multistage pipette puller (Suter Instrument Co., Novato, CA) to pull 1 mm glass capillary
10 tubes into the 6 µm needle configuration. Each micropipette was attached to a MX-110-R 4 axis, manual micromanipulator (Newport Instruments, Newport, CA) using electrode holders. Intracardiac injection of 1 µl of a high titer viral solution (2 x 10⁸) proceeded at a low-flow rate, on the order of 0.2 to 0.5 µl per second (2 to 5 seconds for one
15 microliter.)

The ability to target transgene expression in *in vivo* cardiomyocytes represents a new and powerful approach to study and manipulate specific gene function during the process of cardiac development as well as the treatment of heart disease using gene therapeutic technology. The
20 strategy of using a cardiac-restricted cellular promoter in combination with both the right and left hand ITR sequences from AAV (SEQ ID NO:1 and SEQ ID NO:2, respectively) to achieve cardiac specific transgene expression in both embryonic and post-natal heart tissue distinguishes the present invention from other recombinant adenoviral vectors currently
25 found in the art. Further, the inclusion of both AAV-ITR sequences in the context of a cardiac-restricted recombinant adenovirus vector preserves the tissue-specificity of the cellular promoter activity both *in vitro* and *in vivo* and, when combined with a targeted delivery system, makes the present invention significant as gene based therapy to treat heart disease

as well as providing a method to study specific gene function in embryonic and post-natal heart.

As previously reported in the studies of Fu, *et al.* and Phillip, *et al.*, the presence of AAV-ITR sequences in mammalian cell systems, as well
5 as in developing *Xenopus* embryos, has the effect of enhancing transgene expression. The reports of studies of Fu, *et al.* and Phillip, *et al.* are incorporated herein by reference. While experiments in *Xenopus* embryos suggest that ITR sequences facilitate DNA segregation among replicating cells, other studies implicate AAV- ITR sequences in enhancing genomic
10 integration after transfection, at least in an *in vitro* setting.

Regardless of the mode of action, adenovirus DNA remains mostly in episomal form in infected cells. Since cardiac myocytes, on their own, do not demonstrate robust replication after birth, it is unlikely that these two properties contribute significantly to the enhancement of tissue
15 specificity in heart tissue. An alternative mechanism that has also been implicated in *Xenopus* studies is that AAV-ITR has insulating properties that shield the flanked transgene from the effects of other regulatory elements within the adenoviral genome. In fact, this mode of action has support from findings establishing the existence of negative regulatory
20 elements located around the adenovirus E1a region that can modulate the specificity of the adjacent cellular promoter. Two previous studies from Franz, *et al.* (Cardiovasc. Res. 35, 560-6, 197) and Rothman, *et al.* (Gene Ther. 3, 919-26, 1996) have also reported the generation of cardiomyocyte-specific adenoviruses using the MLC-2v promoter but not
25 with α -MHC promoter even though both promoters have cardiomyocyte-specific transcriptional activity. The reports of studies of Franz, *et al.* and Rothman, *et al.* are incorporated herein by reference. The lack of transgene expression of Adv/CG (CARP promoter without AAV ITR) indicates that the specific transcriptional activity of a cellular promoter is
30 subject to significant influence by the surrounding adenovirus genome.

Therefore, inclusion of AAV ITR provides a general strategy to achieve tissue-specific transcription using other cellular promoters.

Hammond, *et al.* (U.S. Patent No. 5,792,453) have reported a replication defective adenovirus vector comprising a transgene coding for an angiogenic protein or peptide that can be targeted to the myocardium—
5 of a patient by intracoronary injection directly into the coronary arteries, for the treatment of myocardial ischemia. In order to deliver these angiogenic proteins, which may include aFGF, bFGF, FGF-5 (fibroblast growth factors) and VEGF (vascular endothelial growth factor), Hammond,
10 *et al.* rely on ventricular myocyte-specific promoters, namely the promoters from MLC-2v and α -MHC, to achieve targeted delivery. However, as has been established by the method of the present invention, myocardial expression of the angiogenic transgene in the cardiomyocytes is more likely the result of direct cardiac application of the adenoviral
15 vector rather than the use of the MLC-2v or α -MHC promoters. In addition to the CARP gene promoter (SEQ ID NO: 3), the AAV-ITR sequences (SEQ ID NOS: 1 and 2) of the present invention can be used with other cardiac restricted promoters, including:

- 20 1. α -myosin heavy chain gene
2. β -myosin heavy chain gene
3. Myosin light chain 2v gene
4. Myosin light chain 2a gene
5. CARP gene
- 25 6. Cardiac α -actin gene
7. Cardiac m2 muscarinic acetylcholine gene
8. ANF
9. Cardiac troponin C
10. Cardiac troponin I
11. Cardiac troponin T
- 30 12. Cardiac sarcoplasmic reticulum Ca-ATPase gene
13. Skeletal α -actin
14. Artificial cardiac promoter derived from MLC-2v gene

The AAV-ITR sequences can also be used to generate other target vectors for conditional gene expression by using inducible promoters. The
35 inclusion of the AAV-ITR sequences of the present invention, in the

adenoviral vector of Hammond, *et al.* would assure the tissue specific expression of the angiogenic transgene and, thus, avoid the negative effects these angiogenic proteins have on other tissues in the body.

The following examples are intended to illustrate but not limit the present invention.

EXAMPLE 1

Cell-type specific transcription mediated by Adv/CG/ITR vector in cultured cells

This example provides an evaluation of transcriptional specificity of the recombinant adenovirus containing the cardiomyocyte enriched CARP promoter coupled (SEQ ID NO: 3) with the inverted terminal repeat sequences (ITR) from human adeno-associated virus (AAV) (SEQ ID NOS: 1 and 2).

Purified adenoviral vectors were used to infect cultured primary cardiac fibroblasts and ventricular myocytes prepared from neonatal rat heart. An adenovirus vector with a human cytomegalovirus (CMV) enhancer/promoter driving GFP expression (Adv/CMV/GFP) was used as a positive control for viral infection and GFP detection. As previously reported by Wang, *et al.*, *J. Biol. Chem.* 273, 2161-8, 1998, recombinant adenoviruses are capable of efficiently infecting many cell types, including cardiomyocytes, at a low multiplicity of infection (M.O.I.) of less than 100 viral particles/cell and the expression of GFP can be readily detected at a high level in more than 95% of cardiomyocytes cultured from neonatal rat hearts. Cardiac fibroblasts, however, require an M.O.I. of more than 1,000 viral particles/cell in order to achieve approximately 70% of infection. Using the same level of viral infection (100 or 1,000 viral particles/cell), GFP expression was not detected in either myocytes or fibroblasts infected with the Adv/CG vector. In contrast, when the Adv/CG/ITR vector was used as the infecting agent, GFP expression was observed in more than 90% of the cardiac myocytes,

but not at any appreciable levels in cardiac fibroblasts. These results demonstrate that the cardiac specific CARP promoter/AAV-ITR is necessary to achieve transcriptional specificity of the transgene in the ventricular myocytes of cultured neonatal rat heart while transcriptional expression is not found in the fibroblasts even at even high M.O.I.

Further evaluation of cardiac-restricted expression of GFP by Adv/CG/ITR at the transcriptional level was performed using a standard Northern-blot protocol for mRNA detection. As seen in Figure 2, the levels of GFP mRNA in Adv/CMV/GFP infected cardiomyocytes and cardiac fibroblasts are readily detectable. In Adv/CG infected cells, however, the GFP mRNA was not detected, which was in agreement with the observations from evaluation by fluorescent photomicroscopy. In contrast, RNA samples from cardiomyocytes infected with Adv/CG/ITR showed significant levels of GFP transcript, while RNA samples from infected cardiac fibroblasts has significantly lower levels of GFP.

To ensure that the observed cardiomyocyte restricted expression of Adv/CG/ITR vector was at the transcriptional level rather than secondary to an effect of infectivity, a standard Southern-blot analysis was performed using DNA samples from infected fibroblasts and myocytes. As seen in Figure 3, viral DNA was present at comparable levels in both cardiomyocytes and fibroblasts infected with either Adv/CMV/GFP or Adv/CG/ITR vectors. These results confirm that the transcriptional activity of the CARP promoter is suppressed in the context of the adenoviral genome and that the inclusion of ITR sequences from AAV allows retention of cardiac restricted cell-type specificity of the CARP promoter in cultured cells.

EXAMPLE 2

In vivo cardiac restricted transgene expression mediated by the Adv/CG/ITR vector in neonatal mouse heart

In order for the present invention to be viable as a method of gene therapy for the treatment of inherited and acquired heart disease, it is

important to establish that cell type specificity of the Adv/CG/ITR vector, demonstrated *in vitro*, can also direct tissue targeted transgene expression *in vivo*. To test this, approximately 2×10^9 adenovirus particles were injected directly into the heart muscle of day-old mice. Following direct administration of Adv/CMV/ITR vectors into the cardiac cavity, the level of infection was measured to be approximately 10% with a distribution concentrated primarily in the epicardium of the ventricular wall. In addition, a high level of GFP expression was also detected in the liver of the infected animals. This observation agrees with many earlier published studies where it has been established that the delivery of the recombinant adenovirus through the systemic circulation always lead to high levels of infection in the liver and other non-cardiac tissue. Similar to previous observations, direct intracardiac injection of the Adv/CG vector resulted in no detectable GFP in any tissue, including the heart. As predicted, the adenoviral vector of the present invention, Adv/CG/ITR, gave rise to a significant level of GFP expression in heart tissue but a much lower expression in liver and other non-cardiac tissue.

To further evaluate tissue specific expression of the transgene, Northern-blot analyses were performed on RNA samples prepared from the heart and liver of the infected mice. The results of the analysis is shown in Figure 4. In Adv/CMV/GFP injected animals, GFP mRNA was detected at high levels in both the heart and liver confirming the results generated by the Northern-blot analysis. In the Adv/CG/ITR injected mice, however, GFP mRNA was detected primarily in the heart and at a significantly lower level in the liver. The inclusion of AAV ITR in the adenovirus vector, as prescribed in the present invention, enhances the tissue-specificity of transgene expression *in vivo*, making the adenovirus vector of this invention suitable for use in the delivery of gene therapeutic agents.

EXAMPLE 3

Cardiac-restricted transgene expression mediated by the Adv/CG/ITR vector in cultured mouse embryos

- The tissue-specific gene transfer properties of the present invention can also be applied to study gene function during embryonic cardiac development. To demonstrate the ability of targeted gene expression, in developing heart tissue, using tissue specific adenoviral vectors, approximately 2×10^8 particles of each of the recombinant adenovirus vectors, Adv/CMV/GFP, Adv/CG and Adv/CG/ITR were microinjected into the cardiac cavities of developing mouse embryos at 11 days post coitum.
- Following an additional 25 hours of culturing after initial injection of the adenoviral vectors, GFP expression was evaluated. Injection of the Adv/CMV/GFP vector resulted in high relative levels of GFP expression in the developing heart as well as in a wide range of other tissues. This wide spread expression pattern confirms earlier evidence indicating that the Adv/CMV/GFP vector is capable of directing transgene expression in a broad range of tissues and that transgene expression is most likely dictated by the distribution of viral particles in the developing embryo. Following injection of the recombinant Adv/CG vector, analysis by fluorescent photomicroscopy revealed no GFP expression in any part of the embryo which correlated with *in vitro* results derived from cultured cells and *in vivo* data from neonatal mice studies. Injection of Adv/CG/ITR vector gave rise to the expression of GFP in cardiac tissue with no ectopic expression, detectable by fluorescent photomicroscopy, in other tissues. Specifically, GFP expression was at the highest level in the atrium.
- These results demonstrate that inclusion of the ITR sequences from AAV, as in the Adv/CG/ITR vector construct of the present invention, eliminates ectopic expression of the transgene, and allows for cardiac tissue specific expression, following direct ventricular injection of the adenoviral vector into developing embryos. Such tissue specific expression, directed by the Adv/CG/ITR vector of the present invention, can be applied to the development of other recombinant adenoviral vectors that contain ITR sequences from AAV and may confer cardiac

specific expression of a therapeutic transgene in the treatment of cardiac damage and dysfunction.

Although the invention has been described with reference to the examples provided above, it should be understood that various
5 modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims:

What is claimed is:

1. A human type-5 recombinant adenovirus vector which has tissue specific transcription of a transgene, the adenovirus vector comprising;
 - 5 a tissue-restricted promoter; and
 - inverted terminal repeat sequences from human adeno-associated virus (AAV).
2. The human type-5 recombinant adenovirus vector of claim 1, wherein the tissue-restricted promoter is a cardiac-restricted promoter.
- 10 3. The human type-5 recombinant adenovirus vector of claim 1, wherein the tissue specificity is for cardiac tissue.
4. The human type-5 recombinant adenovirus vector of claim 1, wherein the inverted terminal repeat sequences from AAV comprise two copies of the inverted terminal repeat sequence.
- 15 5. The human type-5 recombinant adenovirus vector of claim 4, wherein the two copies of inverted terminal repeat sequence from AAV comprise the left end and right end inverted terminal repeat sequence.
6. The human type-5 recombinant adenovirus vector of claim 5, wherein the left end and right end inverted terminal repeat sequence from
- 20 AAV comprise the 5' end and the 3' end inverted terminal repeats respectively.
7. The human type-5 recombinant adenovirus vector of claim 2, wherein the cardiac-restricted promoter comprises a cardiac-restricted promoter from the group consisting of α -myosin heavy chain gene, β -myosin heavy chain gene, myosin light chain 2v gene, myosin light chain 2a gene, CARP gene, cardiac α -actin gene, cardiac m2 muscarinic acetylcholine gene, ANF, cardiac troponin C, cardiac troponin I, cardiac troponin T, cardiac sarcoplasmic reticulum Ca-ATPase gene, skeletal α -actin, and artificial cardiac promoter derived from MLC-2v gene.

8. The human type-5 recombinant adenovirus vector of claim 7, wherein the cardiac restricted promoter is a cardiomyocyte-restricted ankyrin repeat protein (CARP) promoter.

9. A method for targeted gene therapy for heart disease
5 comprising combining a cardiac-restricted cellular promoter with inverted terminal repeat sequences from adeno-associated virus.

10. The method for targeted gene therapy for heart disease of claim 9, wherein the inverted terminal repeat sequences from AAV comprise two copies of the inverted terminal repeat sequence.

10 11. The method for targeted gene therapy for heart disease of claim 9, wherein the two copies of inverted terminal repeat sequence from AAV comprise the left end and right end inverted terminal repeat sequence.

12. The method for targeted gene therapy for heart disease of
15 claim 9, wherein the left end and right end inverted terminal repeat sequence from AAV comprise the 5' end and the 3' end inverted terminal repeats respectively.

13. The method for targeted gene therapy as in claim 9, wherein the cardiac-restricted promoter comprises a cardiac-restricted promoter
20 from the group consisting of α -myosin heavy chain gene, β -myosin heavy chain gene, myosin light chain 2v gene, myosin light chain 2a gene, CARP gene, cardiac α -actin gene, cardiac m2 muscarinic acetylcholine gene, ANF, cardiac troponin C, cardiac troponin I, cardiac troponin T, cardiac sarcoplasmic reticulum Ca-ATPase gene, skeletal α -actin, and artificial
25 cardiac promoter derived from MLC-2v gene.

14. A method for the evaluation of gene function comprising combining a cardiac-restricted cellular promoter with inverted terminal repeat sequences from adeno-associated virus.

15. The method for the evaluation of gene function of claim 14,
30 wherein the cardiac-restricted cellular promoter is a CARP promoter.

16. The method for the evaluation of gene function of claim 14, wherein the cardiac-restricted cellular promoter is a CARP promoter containing a marker gene.

17. The method for the evaluation of gene function of claim 16,
5 wherein the marker gene comprises a green fluorescent protein gene.

18. The method for the evaluation of gene function of claim 14, wherein the inverted terminal repeat sequences from AAV comprise two copies of the inverted terminal repeat sequence.

19. The method for the evaluation of gene function of claim 14,
10 wherein the two copies of inverted terminal repeat sequence from AAV comprise the left end and right end inverted terminal repeat sequence.

20. The method for the evaluation of gene function of claim 9, wherein the left end and right end inverted terminal repeat sequence from AAV comprise the 5' end and the 3' end inverted terminal repeats
15 respectively.

1/2

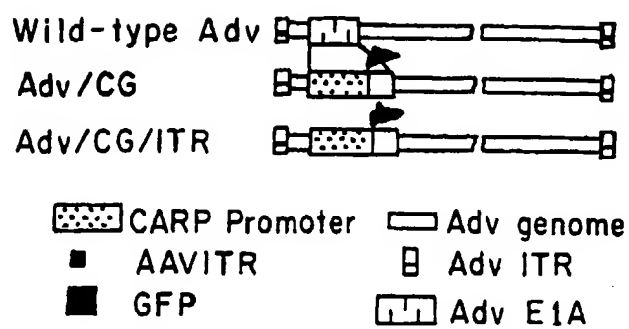


FIG. 1

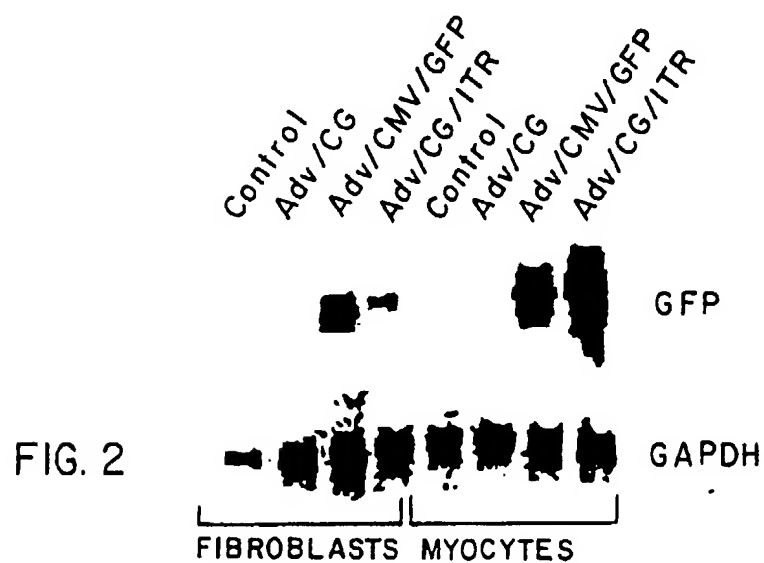


FIG. 2

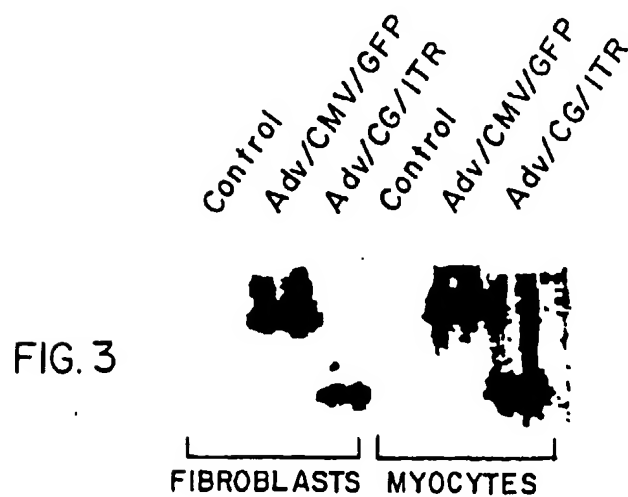


FIG. 3

2/2

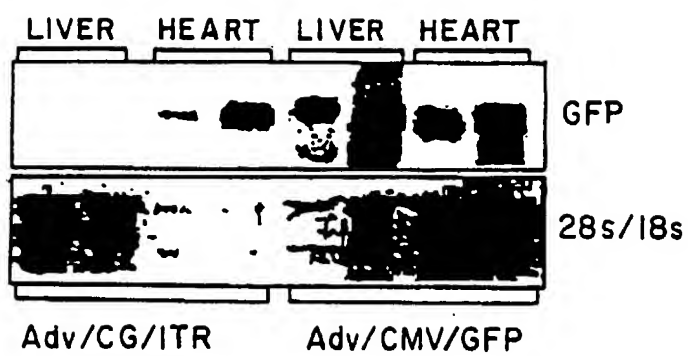


FIG. 4

SEQUENCE LISTING

<110> Kenneth Chien
 Yibin Wang
 Sylvia Evans
 <120> NOVEL RECOMBINANT ADENOVIRUS FOR TISSUE
 SPECIFIC EXPRESSION IN HEART
 <130> 6627-8045
 <140> unknown
 <141> September 10, 1999
 <150> US 60/099,960
 <151> September 11, 1998
 <160> 3
 <170> Word Perfect 8.1

 <210> 1
 <211> 174
 <212> ssDNA
 <213> adeno-associated virus 2; Viruses; ssDNA viruses;
 Parvoviridae; Parvovirinae; Dependovirus

 <220>
 <221> enhancer; 5' inverted terminal repeat
 <222> 1...174

 <400> 1
 ggccactccc tctctgcgcg ctcgctcgct cactgaggcc gcccgggcaa 50
 agcccgggcg tcgggcgacc ttggtcgcc cggcctcagt gagcgagcga 100
 gcgcgcagag agggagtggc caactccatc actaggggtt cctggagggg 150
 tggagtcgtg acgtgaatta cgta 174

 <210> 2
 <211> 183
 <212> ssDNA
 <213> adeno-associated virus 2; Viruses; ssDNA viruses;
 Parvoviridae; Parvovirinae; Dependovirus

 <220>
 <221> enhancer; 3' inverted terminal repeat
 <222> 1...183

<400>	2				
catggctacg	tagataagta	gcatggcggg	ttaatcatta	actacaagga	50
acccttagtg	atggagttgg	ccactccctc	tctgcgcgct	cgctcgctca	100
ctgaggccgg	gcgaccaaag	gtcgcccgac	gcccgggctt	gcccgggcg	150
gcctcagtga	gcgagcgagc	gcgcagagag	gga		183

<210>	3
<211>	2247
<212>	mRNA
<213>	Mus musculus; Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Mammalia; Eutheria; Rodentia; Sciurognathi; Muridae; Murinae; Mus.

<220>	
<221>	promoter
<222>	1-2247

<300>	
<301>	Zou, Y., et al.
<302>	CARP, a cardiac ankyrin repeat protein, is downstream in the Nkx2-5 homeobox gene pathway
<303>	Development
<304>	124
<305>	4
<306>	793-804
<307>	1997

<400>	3				
nagctencat	gcctgcaggt	cgactctaga	ggatcctttc	atgtttaaca	50
atatcaacc	taaccaagg	ggaacagcct	gcctgacagt	ggctttgcc	100
cccatgaata	cttcctagtc	tagtccgttt	gtgaaactca	gcccattcca	150
acacttctgc	aagccccatc	ctctacaagg	tgctcattgg	gaatttcctg	200
gagcttctct	ttcaggatca	gcctgattct	agggcagcag	ttctcaacct	250
gggggcctcg	accctttgg	gggaatcaaa	cgacccttta	caggggtcac	300
atatcatcta	tcctatatgt	caggtattta	cattacgatt	cgtaacagta	350
gcaaaattac	aggtatgaaa	tagcaatgaa	ataattttat	gattgaaggt	400
caccacaaca	tgaggccgcc	acactgttct	agagaaaaat	cacctgggtg	450
gggaaaggtt	tggaagacc	ttctgtcca	ttcttcattc	ttcaaagtga	500
tgtgttcaca	gaaagccttt	cagctgttct	gctggggctc	ttagtaagtc	550
tgagtaggaa	ctgtatgtac	caggtctgct	tcttatgggt	ggagccaaga	600
cgcatcgtgg	gtggagcgaa	gacgcaacct	caccttctac	tctgcacca	650
tagcaagtag	cctaattgtc	tgngtctagg	gtcatctctg	tgaatcgaga	700
tccttgccc	ttgtttgaat	tagggaggca	caaaatctta	aaaaattcaa	750
gactgntcaa	caanccanaa	gtcctttctc	aaaaggaaag	gncttaactn	800

tnanccccc	tttactttg	agtcaaggcc	tggaaccaa	ccggccccag	850
gaatgaaaa	agcttgccat	nacctggtg	gcccccttna	anaggncaaa	900
aaaaaattgt	ggttaacntt	gaaaaaccga	agaccaacag	ttatcctcta	950
gaaacacaat	ttgctgggtg	aacagctgaa	gtggggtggg	ggttcttacc	1000
ccatgttcat	ggaagggtga	gtgaggagag	acagatatat	gaggccagca	1050
taacaaacat	acacaacacc	ctaattaaca	cttccctctt	ctactgacac	1100
cccccttca	ctcctcttct	ataaaaaata	aaaaaagtat	tttagtggct	1150
cttacgatag	aatctttcct	cgaactataa	aaagatctaa	atatttatat	1200
ttttcacatt	ttaatatctt	agcgatgaca	agccagaaac	aagatttttt	1250
gcctctctca	acagcaaagc	ttggggcctt	ttgtttccg	tgtagggaat	1300
agaacacgag	agccccgtgt	atctaggcag	atgctctatc	attagcccat	1350
gagtctccag	cctcagacgc	acattttctt	cgggctctct	taagcttttc	1400
ccacagcatt	gggaaacttt	actgacagca	tccaagtgtg	gctctgcta	1450
agaactggac	tcacatctct	ctggcatcac	ttcggcccgt	tttgggtag	1500
atcctctgat	tagccttcag	atttagaaca	cggtagacct	gtggtcacta	1550
attatggcca	gtgacacat	agagtcaaag	tgcattactg	aatgctttca	1600
atttctccta	atgctggtac	gatggcatgt	cacagggcca	tttagctgc	1650
agacatcatc	cagagaattc	caaacagata	ggacaagtgg	caccagacc	1700
catctccttc	ccctcgggct	gattatcccc	aaaataggat	gtcccaaagc	1750
aacacttccc	agccaactgg	agtgtgata	agtccagtta	tcagaaagat	1800
atggctgtaa	gtgtgatgca	cagtgttgc	atttcttga	tacgttagtc	1850
atatgagagc	tgacaaagaa	ggaaaaagag	cagcgatgtg	tgcaatatta	1900
acaggcagct	gtccccctggc	ttcccgatac	gtgggatgac	tcgcattgct	1950
gagcgggtgtg	gtcactgcca	aagggaatgac	cctctcacat	ttcttctga	2000
ttgcatacg	ccgcggccag	cttgtcatct	ccctcttggg	cttcccagac	2050
actaagtctg	gaatgaaaat	tcacctgcct	ctgaattggc	cactggtggg	2100
agcaggggtg	tgacttggct	tcccaggctg	gaagattatc	tcaccagacc	2150
ctactatata	acgggctggt	gtggaggggc	tccacagggc	cagttccagg	2200
ggttcatcca	caagagagaa	aaacatagac	tcacggctgc	caacatg	2247

INTERNATIONAL SEARCH REPORT

Intern. Application No

PCT/US 99/20730

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>WO 98 10088 A (UNIV PENNSYLVANIA) 12 March 1998 (1998-03-12)</p> <p>page 4, line 30 -page 14, line 18; claim 26</p> <p style="text-align: center;">---</p>	1,4-6, 9-12, 17-20
A	<p>WO 94 11506 A (ARCH DEV CORP) 26 May 1994 (1994-05-26) the whole document</p> <p style="text-align: center;">---</p>	1,9,14
A	<p>PHILIP R ET AL: "EFFICIENT AND SUSTAINED GENE EXPRESSION IN PRIMARY T LYMPHOCYTES AND PRIMARY AND CULTURED TUMOR CELLS MEDIATED BY ADENO-ASSOCIATED VIRUS PLASMID DNA COMPLEXED TO CATIONIC LIPOSOMES" MOLECULAR AND CELLULAR BIOLOGY, vol. 14, no. 4, April 1994 (1994-04), page 2411-2418 XP000676937 ISSN: 0270-7306 cited in the application the whole document</p> <p style="text-align: center;">---</p>	1,6
A	<p>ZOU Y ET AL., : "CARP, a cardiac ankyrin repeat protein, is downstream in the Nkx2-5 homeobox gene pathway" DEVELOPMENT, vol. 124, 1997, pages 793-804, XP000863100 cited in the application the whole document</p> <p style="text-align: center;">---</p>	8,15
A	<p>JEYASEELAN R ET AL., : "A novel cardiac-restricted target for doxorubicin" THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 272, no. 36, 5 September 1997 (1997-09-05), pages 22800-22808, XP002125498 cited in the application page 22803, right-hand column, paragraph 3; figure 4 discussion.</p> <p style="text-align: center;">---</p>	8,15
A	<p>YEH P AND PERRICAUDET M: "Advances in adenoviral vectors: from genetic engineering to their biology" FASEB JOURNAL, vol. 11, no. 8, 1997, page 615-623 XP002086861 ISSN: 0892-6638 cited in the application the whole document</p> <p style="text-align: center;">-----</p>	1

INTERNATIONAL SEARCH REPORT

information on patent family members

Intern. Application No

PCT/US 99/20730

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9613598 A	09-05-1996	US 5856152 A	05-01-1999
		AU 695811 B	20-08-1998
		AU 4405596 A	23-05-1996
		CA 2203808 A	09-05-1996
		EP 0797678 A	01-10-1997
		JP 10507928 T	04-08-1998
		US 5871982 A	16-02-1999
WO 9810088 A	12-03-1998	AU 4183397 A	26-03-1998
		EP 0931158 A	28-07-1999
WO 9411506 A	26-05-1994	AU 694097 B	16-07-1998
		AU 5609394 A	08-06-1994
		CA 2149771 A	26-05-1994
		EP 0668913 A	30-08-1995
		EP 0957172 A	17-11-1999
		JP 8506008 T	02-07-1996